The unidirectional hypoxia-activated prodrug OCT1002 inhibits growth and vascular development in castrate-resistant prostate tumours.

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Running Title: Effect of OCT1002 on hypoxic tumours

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ABSTRACT

Background. OCT1002 is a unidirectional hypoxia-activated prodrug (uHAP) OCT1002 that can target hypoxic tumour cells. Hypoxia is a common feature in prostate tumours and is known to drive disease progression and metastasis. It is therefore a rational therapeutic strategy to directly target hypoxic tumour cells in an attempt to improve treatment for this disease. Here we tested OCT1002 alone and in combination with standard-of-care agents in hypoxic models of castrate-resistant prostate cancer (CRPC).

Methods. The effect of OCT1002 on tumour growth and vasculature was measured using murine PC3 xenograft and dorsal skin fold (DSF) window chamber models. The effects of abiraterone, docetaxel and cabazitaxel, both singly and in combination with OCT1002, were also compared.

Results. The hypoxia-targeting ability of OCT1002 effectively controls PC3 tumour growth. The effect was evident for at least 42 days after exposure to a single dose (30mg/kg) and was comparable to, or better than, drugs currently used in the clinic. In DSF experiments OCT1002 caused vascular collapse in the PC3 tumours and inhibited the revascularisation seen in controls. In this model OCT1002 also enhanced the anti-tumour effects of abiraterone, cabazitaxel and docetaxel; an effect which was accompanied by a more prolonged reduction in tumour vasculature density.

Conclusions. These studies provide the first evidence that OCT1002 can be an effective agent in treating hypoxic, castrate-resistant prostate tumours, either singly or in combination with established chemotherapeutics for prostate cancer.

Keywords: OCT1002, hypoxia-activated pro-drug, prostate, hypoxia, castrate-resistant, PC3

INTRODUCTION

Hypoxia occurs in most solid tumours and it is known to have a major influence on treatment response to radiotherapy[1], chemotherapy[2] and immunotherapy.[3] Untreated prostate tumours are known to be very hypoxic (~0.3% oxygen)[4,5], which is >12 times lower than oxygen levels found in the normal prostate (~4% oxygen).[6] High levels of hypoxia significantly correlate with increasing clinical stage and can predict biochemical failure following radiotherapy.[7,8] Furthermore, hypoxia has also been implicated as a causative factor in malignant progression[9], genetic instability[10], gene amplification[11], endothelial-to-mesenchymal transition (EMT)[12,13] and selection of cells with diminished apoptotic potential and a greater invasive potential.[14,15] It is therefore a viable therapeutic strategy to directly target hypoxic tumour cells in an attempt to improve treatment, meaning hypoxic targeting is likely to be a key part of precision medicine for prostate cancer.[16,17]

One approach to blocking the influence of hypoxic tumour cells is to directly target this sub-population with a hypoxia-activated prodrug (HAP). HAPs are compounds that are designed to be reduced to an active, toxic form in cells when oxygen levels are very low.[18,19] However, conventional HAPs (e.g. tirapazamine, TH302, PR-104) are reduced in single-electron reduction steps, a reversible process that can redox cycle producing reactive oxygen in normoxic tissue.[16] In contrast, alkylaminoanthraquinone-di-N-oxides are prodrugs that have a different mechanism of activation. These aliphatic N-oxides are reduced through an obligate two-electron reduction. This results in oxygen atom transfer which under hypoxic conditions results in irreversible formation of a stable anti-proliferative agent and water. For this reason, our previous work has focused on AQ4N[20] and its more recently described deuterated analogue OCT1002 (OncoTherics Ltd).[21,22] These *unidirectional* HAPS (uHAPs) are reduced in hypoxia to the metabolically stable reduction products (AQ4 and OCT1001, respectively). Studies with AQ4N and OCT1002 show that hypoxia-mediated reduction results in a product with high affinity for DNA and targeting of topoisomerase II, [23,24]which can effect a long-term inhibition of both DNA replication and G2/M cell transition.[25,26]

Previously we have shown that OCT1002 kills hypoxic prostate cancer cells *in vitro* and *in vivo*.[21] We provided the first evidence that OCT1002 has a hypoxia-dependent anti-tumour effect in androgen-sensitive LNCaP prostate tumour xenografts and the effect can be markedly enhanced when combined with daily bicalutamide administration, a drug which targets the androgen receptor (AR). This is consistent with previous studies in the same model, which showed that AQ4N can block re-oxygenation that occurs during prolonged (> 14 days) treatment with daily bicalutamide.[27] We have also shown that single-modality hormone treatment drives development of more malignant tumours, a consequence of reduced vascularisation and oxygenation (days 1-14) followed by a revascularisation and re-oxygenation over the next 14 days. This was accompanied by a cascade of molecular and phenotypic changes that included evidence of EMT and increased metastasis to the lungs within 4 weeks.[13,27] The novel analogue OCT1002 caused similar effects in LNCaP xenografts and we also showed that it could block significantly the molecular changes caused by bicalutamide alone,[21] thereby demonstrating that detrimental hypoxia-induced cellular responses can be effectively blocked.

To further demonstrate the potential of OCT1002, it is important to test its effect in other xenograft models to ensure this is not a cell-line specific result. In particular, it would be instructive to see its impact on androgen-independent prostate tumour cells, since there is a clear clinical need for strategies that can improve the treatment of castrate-resistant prostate cancer (CRPC). Therefore, the current study has been designed to evaluate the effect of OCT1002 on CRPC tumour growth using murine PC3 xenograft and dorsal skin fold (DSF) models. The DSF model allows the extra opportunity to analyse changes in tumour vasculature within the tumour microenvironment in response to drug interaction. For comparison, the effects of three drugs currently used in the clinic to treat CRPC (abiraterone, docetaxel and cabazitaxel) are similarly investigated, singly and in combination with OCT1002.

MATERIALS AND METHODS

Cell Culture

All cell-lines were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were frozen at low passage number and used within 3-6 passages. Cells were authenticated by in-house genotyping service and routinely confirmed as mycoplasma-free (InvivoGen, Toulouse, France). LNCaP cells were cultured in RPMI 1640 culture medium (Life Technologies, Paisley, UK) supplemented with 10% foetal bovine serum (FBS), D-glucose (10mM; Sigma, Poole, UK) and HEPES (10mM; Sigma). PC3 and 22Rv1 were cultured in RPMI-1640 supplemented with 10% FBS. For treatment in hypoxic conditions, 5,000 cells were seeded in a 96 well plate and allowed to adhere overnight. After dosing with vehicle (phosphate buffered saline, PBS) or OCT1002 (1μM), cells were grown at 37°C in 0.1% oxygen in a Invivo$_2$ hypoxia work station (Ruskinn Technology, Bridgend, UK) for 0, 4, 24, 48 or 72 hours, before being placed in normoxia (20% oxygen) for 72, 68, 48, 24 and 0 hours. The cells were then harvested and cell viability was measured using an XTT assay (Roche, Sussex, UK).

In vivo methods

Animal maintenance. In vivo experiments were conducted in accordance with the Animal (Scientific Procedures) Act 1986 and the UKCCCR guidelines for the welfare of animals in experimental neoplasia.[28] Male athymic nude mice (8 – 10 weeks) weighing 30-35g (Envigo, Cambridgeshire, UK) were housed under standard laboratory conditions in a temperature-controlled (22°C; 50-55% humidity) pathogen-free environment with a 12 hour light-dark cycle. Food and water was supplied *ad libitum*.

Xenograft establishment. Tumour oxygenation and growth delay. LNCaP, 22Rv1 and PC3 xenografts were established on the rear dorsum of nude mice by subcutaneous injection of 2 x 10^6 cells suspended in 100μl of matrigel with a 21g needle (Becton Dickinson, Oxford, UK). . When tumours

reached approximately 200mm^3 intra-tumour pO_2 was measured using an OxyLiteTM 2000E system (Oxford Optronix, UK) as previously described.[13,27] For growth delay studies, tumours were measured every 2 days using Vernier callipers. When the tumour volume reached between 150-200mm^3, mice were randomly assigned to treatment groups and dosing initiated (day 0). Mice were sacrificed on day 28 in the 4-week dosing experiment and on day 42 in the 2 week dosing experiment except in the cabazitaxel group, which required humane sacrifice on day 35 due to toxicity.

Drug administration. OCT1002 (OncoTherics, Shepshed, UK) was prepared in sterile PBS and administered by intraperitoneal (i.p.) injection (30mg/kg; once per experiment unless otherwise indicated). Cabazitaxel (5mg/kg; once per week) (Hangzhou Dayang Chemical Industry Limited Company, Zhejiang Sheng, China), docetaxel (10mg/kg; twice per week) (Sigma) were administered by i.p. injection. Abiraterone (98 mg/kg; daily) (Hangzhou Dayang), was administered orally (p.o.) by gavage. Vehicle was 0.1% DMSO in corn oil (Sigma).

Dorsal skin fold model

Dorsal skin fold (DSF) preparation. A bespoke transparent DSF 'window chamber' (APJ Trading Co. Ltd, USA) was attached to the dorsum of anaesthetized nude mice. A tumour fragment (roughly 0.5mm in diameter, obtained from a mouse PC3 xenograft, was implanted onto surgically exposed panniculus carnosus muscle within the window, washed with saline and covered with a plastic cover slip. After surgery mice were given a topical prophylactic antibiotic (Chloramphenicol; Martindale Pharmaceuticals, UK). Tumours were allowed to vascularize for 7 days following surgery and then randomly assigned to treatment groups, this was designated as day 0, and assigned to treatment groups with drug concentrations as described above. Tumour vasculature was then imaged on days 0, 7, 14, 21 using a stereomicroscope. Image analysis was carried out using Touptek software (Touptek Photonics, China). All surgical procedures were performed under aseptic conditions and the body temperature of animals was kept constant using heated pads.

Statistical analysis

Data from *in vitro* and *in vivo* studies were analysed using a two-tailed student's t-test unless otherwise indicated. All statistical analysis was carried out using the Prism 5.0 software (GraphPad). Differences between points were deemed statistically significant with a $p<0.05$ (95% confidence interval).

RESULTS

Effect of OCT1002 on prostate cancer cell growth in hypoxia

Initially, *in vitro* studies were carried out in 3 prostate cancer cell lines (LNCaP, 22RV1 and PC3) to confirm their potential for differential cytotoxicity of OCT1002 in normoxia (20% oxygen) and hypoxia (0.1% oxygen). After as little as 4 hours exposure to hypoxia, OCT1002 (1 μM) decreased the viability of cells as compared to normoxia in all cell lines (Supplementary Figure 1). At most time points (4, 24, 48 and 72h) OCT1002 caused more cell death than hypoxia alone.

OCT1002 slows tumour growth control in hypoxic PC3 xenograft model

When prostate tumour xenografts were grown *in vivo* in nude mice, they showed a reproducible and consistently low level of oxygenation that was characteristic of each cell line; LNCaP: 14.5 ± 2.02 mmHg (1.9% oxygen), 22Rv1: 11± 0.74 mmHg (1.4 % oxygen) and PC3: 3.7 ± 0.71 mmHg (0.49% oxygen) (Figure 1A). The toxicity of OCT1002 in nude mice was assessed by measuring their weight for 21 days after administration of a single dose of OCT1002 (10 or 30 mg/kg). No significant difference in weight was observed in either treatment group compared to control. All groups showed a small but steady increase in weight throughout the observation period confirming the lack of toxicity of OCT1002 in nude mice up to at least 30mg/kg (Figure 1B).

Subsequently, nude mice bearing PC3 tumours were treated with OCT1002 (30mg/kg; day 1 or days 1 and 7) over 28 days (Figure 1C). For comparison three drugs widely used in late stage CRPC, at dosing schedules reported in preclinical studies i.e. cabazitaxel (5mg/kg 1x week), abiraterone (98

mg/kg daily) and docetaxel (10mg/kg 2x week). The effects of all drug treatments on tumour growth were compared to vehicle. OCT1002 as a single low dose treatment showed a marked effect on this severely hypoxic tumour; adding a second dose at day 7 did not have any additional effect. Of the three other chemotherapy drugs, abiraterone showed the highest anti-tumour effect whereas docetaxel and cabazitaxel displayed a growth control very similar to OCT1002. It is interesting that abiraterone was markedly effective against PC3 tumours, which are AR-negative, since its primary action is known to be blockade of androgen synthesis through inhibition of CYP17A1. However, several *in vitro* studies have demonstrated that the anti-tumour effect of abiraterone in PC3 cells is not solely associated with blockade of androgen synthesis.[29-31] Our *in vivo* studies would confirm these observations.

In a second experiment, tumours were treated only with the selected regimens for 14 days and the effect of the drugs on tumour growth was followed for a further 28 days (Figure 1D). Regrowth of the tumour was considerably more marked for cabazitaxel and this drug caused considerably more toxicity, to the extent that by day 35 all of the animals in this group had been sacrificed. The other treatment regimens were well tolerated until day 42 when the experiment was ended. The single dose of OCT1002 (30mg/kg on day 1) was as effective as the 14-day scheduled dosing of abiraterone and docetaxel in controlling tumour regrowth. Representative images of mice and the excised tumours are provided in Figure 1E.

OCT1002 inhibits tumour growth and reduces PC3 tumour vasculature in DSF model

PC3 tumour fragments were grown in DSF 'window chambers' and these were used to measure the effect of OCT1002 on tumour growth and vasculature. Abiraterone, cabazitaxel and docetaxel were also tested for comparison. All drugs had some effect on tumour growth in comparison to vehicle treatment, with abiraterone being the most effective, showing a particularly large anti-tumour effect between days 14 and 21 (Figure 2A and 2B). Vasculature was assessed by calculating the mean density of tumour blood vessels, which markedly increased for control tumours from day 0 to day 21

(Figure 2C and 2D). OCT1002 treatment resulted in a significant reduction in the mean vessel area covered on days 14 and 21, while abiraterone resulted in a significant vessel reduction at days 7, 14 and 21. Docetaxel significantly reduced vasculature compared to vehicle on day 7 and 14, but by day 21 tumour vasculature was restored to levels similar to that displayed in control tumours. Cabazitaxel treatment showed a trend towards reduced vasculature compared to control but these measurements did not reach significance.

Effect of drug combinations on PC3 tumour growth and vasculature

Each of the clinically approved chemotherapy drugs was then given in combination with OCT1002 on day 1. In the PC3 xenograft model, no marked advantage was observed in the combinations, although it was noted that cabazitaxel was better tolerated when the mice were treated with cabazitaxel in combination with OCT1002 (Figure 3A-3C). However, when tumours were analysed in the DSF model, OCT1002 caused a significant growth reduction when used in combination with the other drugs, compared to single treatment (Figure 4A-4C). This effect was most marked when OCT1002 was used in combination with cabazitaxel and docetaxel (Figure 4B and 4C).

When the mean vessel density was measured in the PC3 DSF model it was clear that the drug combinations also had an effect on tumour vasculature. When OCT1002 was combined with abiraterone the vascular density was further reduced on day 14 and 21, compared to OCT1002 alone (Figure 5A). OCT1002 appeared to cause some further reduction on the vasculature in combination with cabazitaxel, though it only reached significance on day 14 (Figure 5B). Combining OCT1002 with docetaxel resulted in significantly reduced vasculature at day 14 and day 21 compared to either drug used singly (Figure 5C).

DISCUSSION

Improving treatment of CRPC remains a major challenge for clinicians. Current chemotherapy includes cytotoxic agents such as docetaxel and cabazitaxel, or drugs targeting AR axis signalling, such as enzalutamide and abiraterone.[32] However, drug resistance to these agents is common, meaning novel strategies and/or combinations are required to combat this.[32,33] Since hypoxia is a common feature of prostate tumours and a known driver of prostate cancer progression, the use of HAPs/uHAPs to target hypoxic cells represents a novel approach which can address developing drug resistance. This study presents further pre-clinical evidence to demonstrate the potential of the uHAP OCT1002 as a novel drug for treating prostate cancer.

We first confirmed OCT1002 was capable of killing prostate cancer cells grown in hypoxic conditions *in vitro* (Supplementary Fig 1) to corroborate cell-line analysis from our previous study.[21] Having demonstrated the effect of OCT1002 on PC3 cells *in vitro*, we proceeded to use PC3 cells in murine models *in vivo*. In our previous work, we measured the oxygen levels in LNCaP and 22Rv1 tumours in SCID mice and found that each untreated tumour type had an intrinsic level of oxygenation that was specific and consistently reproducible.[13,21] Here, we demonstrated that PC3 tumours grown in nude mice are considerably more hypoxic than LNCaP and 22Rv1 tumours (Figure 1A). Based on our experience of tumour xenograft models, we propose that the intrinsic hypoxic status of the tumours is determined by several inter-dependent factors. Clearly a major factor will be the extent of the vascular network that develops *in vivo,* which can subsequently change in response to anti-tumour treatments.[13,21] Linked to this, these prostate tumour cell lines have a varying genetic background and exhibit considerable differences in a number of signalling pathways that affect both metabolism and the cells' ability to develop a functioning vascular network.[34] Thus, it is not surprising that they exhibit differing levels of vasculature and oxygenation in the tumours grown *in vivo*. Furthermore, it is likely that tumour hypoxia will vary depending on the mouse strain used in these models. For example, we get consistent results for each model that we have used, but interestingly LNCaP

tumours are more hypoxic when grown in SCIDs than nudes, suggesting the specific characteristics of the growth environment plays a role too. It therefore appears that oxygenation of tumours *in vivo* is determined by different over-lapping physiological and molecular influences, which will be important to our understanding of tumour biology in the treatment responses of preclinical models. Indeed, many of these factors are likely to influence treatment responses for CRPC.

The intrinsically low oxygen levels in PC3 tumours suggested that OCT1002 should certainly be activated in these tumours. Indeed, it should be noted that all three of these xenograft tumours have oxygen levels which are well below that of normal prostate tissue ~4%. The range demonstrated in the mouse tumour xenografts is consistent with oxygenation levels exhibited by human prostate tumours, making them a clinically relevant model for testing OCT1002.[6]

We first showed a lack of OCT1002 systemic toxicity in this PC3 xenograft model, as measured by mouse body weight retention (Figure 1B). This is consistent with OCT1002 acting as a prodrug which is inactive in normal tissues. We then proceeded to test its ability to control tumour growth, using three approved chemotherapy drugs for comparison purposes. All drugs demonstrated significant tumour growth control at day 28 compared to vehicle treatment. Cabazitaxel (5mg/kg; 1x week) had some anti-tumour efficacy, but was also the most toxic to the mice. Docetaxel (10mg/kg; 2x week) showed a similar inhibition of tumour growth but was better tolerated. Abiraterone (98 mg/kg; daily) exhibited the best tumour growth control. Comparing these drugs with OCT1002, we found it particularly noteworthy that a single, low dose of OCT1002 (30mg/kg; administered on day 1) demonstrated similar tumour growth control to both cabazitaxel and docetaxel (Figure 1C). As observed above, this may be because PC3 tumours are extremely hypoxic and thus particularly susceptible to OCT1002. This also underlines the potential success that OCT1002 may have in the clinic if used in patients identified to have hypoxic tumours, either by molecular or physical biomarkers. Encouragingly, PET- based imaging techniques have been established that can be used to quantify the hypoxic fraction,[35] including an on-going clinical trial (NCT01567800), which could

identify those patients most likely to benefit from OCT1002. For example, CRPC patients predicted to have worse outcome on enzalutamide or abiraterone[36] could be offered a new treatment option based on use of OCT1002.

The persistent, long-lasting effect of OCT1002 was further demonstrated when we stopped treatment with the three approved drugs at day 14 and compared tumour regrowth over the next 28 days to the OCT1002 treatment group, which had received a single dose on day 1. This single, early dose of OCT1002 showed a similar ability to inhibit tumour regrowth as abiraterone and docetaxel (Figure 1D and 1E). It was markedly better than cabazitaxel treatment, which proved quite toxic to mice in this experiment, to the extent that mice had to be prematurely sacrificed. This demonstrates the unique uHAP properties of OCT1002, in that a long-lasting effect is achieved due to the irreversible activation within hypoxic cells to the toxic reduction product OCT1001. We propose this selective targeting prevents the ability of hypoxia-resistant cells to re-establish the tumour as quickly.

Since our previous work had identified the importance of drug effects on LNCaP tumour vasculature, we wanted to extend this to other tumour models and therefore examined how OCT1002 impacted upon PC3 tumour vasculature *in vivo*. We compared the effects of OCT1002 and the three clinically approved drugs on tumour growth and vasculature using the DSF window chamber assay (Figure 2). As was observed using the dorsal tumours, a single dose of OCT1002 (30mg/kg; administered on day 1), demonstrated similar tumour growth control to both cabazitaxel and docetaxel (Figure 2A and 2B). Abiraterone was again the most effective in controlling tumour enlargement in the window chamber.

As for tumour vasculature, all drugs demonstrated a significant effect compared to vehicle-treated tumours at various time intervals following dosing (Figure 2C and 2D). OCT1002 resulted in significantly reduced tumour vasculature density at day 14 and 21, compared to control tumours, an effect we had previously noted in LNCaP xenografted tumours.[21] In contrast, docetaxel showed a

marked anti-vascular effect at day 7 and day 14, but by day 21 the vasculature had recovered to levels displayed by control tumours, despite continuance of the twice weekly treatment. Cabazitaxel treatment reduced vasculature, but not significantly. Abiraterone proved to have the most consistent effect, resulting in significant reduction of vessel coverage compared to control tumours from day 7 to 21. This corroborates a similar anti-vascular effect in LNCaP tumours treated with bicalutamide in DSF experiments.[13,21,27] It is likely that this is a consequence of the blockade of tumour cell growth, which will disrupt its capacity for pro-angiogenic signalling and hence vessel formation. However, our previous work also demonstrated that tumour vasculature could re-establish itself eventually (after 14 – 21 days), even with continued dosing. We hypothesised that resistant tumour cells which survived the increased hypoxic stress caused by vascular reduction were able to promote re-growth of the tumour vasculature. This new data provides further evidence for this, in the observation that the initial inhibitory effect of docetaxel on blood vessels was abrogated by day 21, which is very similar to the effect we noted using bicalutamide against LNCaP tumours. This ability of tumours to recover quite quickly from chemotherapeutic interventions is a worrying observation as it suggests that resistant cells within tumours adapt to treatment by adopting a more pro-angiogenic phenotype, In LNCaP tumours we have shown that this adaptation was accompanied by changes associated with development of a more malignant genotype.[13,21] This would help to explain why patients frequently relapse to a more aggressive disease following initially successful therapy. To address this, it seems clear that strategic drug combinations are required to overcome resistance, either concomitantly or sequentially. Indeed, recent results from the CHAARTED[37] and STAMPEDE[38] clinical trials have revealed that docetaxel in combination with androgen deprivation therapy improved relapse-free survival in patients with high-risk localised prostate cancer. A more recent report from the STAMPEDE trial also showed that men with locally advanced or metastatic prostate cancer who received ADT plus abiraterone and prednisolone had significantly higher rates of overall and failure-free survival than those who received ADT alone.[39] Hence the right drug combinations can overcome tumour resistance in prostate cancer sufferers. As

hypoxia is widely recognised to play a crucial role in developing tumour resistance to therapy, the current study supports the use of for OCT1002 as a strategic drug for use in such a combination.

To further investigate this we therefore examined the effect of combining OCT1002 with agents clinically used in prostate cancer on PC3 tumour growth. We could detect no additional benefit of these combinations on xenograft tumour growth inhibition using the particular schedules chosen. However, as we have shown PC3 tumours are very hypoxic so we predicted we might get a maximal effect with OCT1002 as a single agent. Previously in a pilot study of another intrinsically very hypoxic xenografted pancreatic tumour (0.3% oxygenation in control tumours) we found a marked effect equivalent to treatment with the standard-of-care drug gemcitabine (unpublished data). This confirms to us that when assessing uHAP effects in a tumour that is intrinsically very hypoxic then significant effects can be achieved even with single agent therapy, meaning the scope for seeing an additive effect is limited. Another important consideration in assessing drug combinations is the potential for negative interactions. However, our findings provide a firm basis for excluding negative interactions between OCT1002 and other standard-of-care drugs. This will enable combinations to be explored to identify the potential for additivity or even synergy in dose schedule studies.

In the current study we felt that, before progressing to further scheduling combinations, it was important to look for effects in the DSF model since we have found this model to be more sensitive and capable of showing subtler differences. Using this approach, we did observe that OCT1002 can enhance the anti-tumour effects of the three other drugs. Abiraterone was very effective as a single agent at tumour growth inhibition in this model but never-the-less a statistically significant improvement was noted at day 7 and day 14 when it was used in combination with OCT1002. The combination was also significantly better at controlling tumour growth than OCT1002 alone at day 7 to 21. Similarly, combining OCT1002 with cabazitaxel was significantly better at controlling tumour growth than either treatment alone at day 7 to 21. Finally, combining OCT1002 with docetaxel was significantly better at controlling tumour growth than either treatment alone at day 14 and day 21.

When we looked at the effect of the combinations on vasculature density, the most interesting effect was for docetaxel (Figure 5C). At day 14 and day 21, the combination showed a significant reduction of vasculature compared to either treatment alone. At day 21, the restoration of vascular density observed with docetaxel alone was completely abrogated when combined with OCT1002. This corresponds with the inhibitory effect of the docetaxel/OCT1002 combination on tumour growth observed in this model, showing promise for this treatment combination in the clinic for CRPC. Indeed, the low toxicity of OCT1002 and its mode of action may help address issues of polypharmy and toxicity associated with treatment of metastatic CRPC patients[40] by allowing lower doses of standard chemotherapy drugs to be potentially used.

Overall, our results demonstrate that OCT1002 has potential for treating CRPC. Further work is now needed to probe the specific effects of OCT1002 at a molecular and cellular level, as well as optimising the dosing and scheduling of OCT1002 as a single agent or in combination with other drugs. Furthermore, it is now clear that a wide molecular diversity exists in human prostate tumours.[41] Understanding the complexities of this diversity is important for patient stratification as different sub-types of prostate cancer exist, each with different druggable pathways which may be potentially targeted.[41,42] In OCT1002, we have an agent that when activated inhibits topoisomerase II,[24] a critical player in DNA replication, DNA repair and AR signalling,[43] therefore impacting upon targets that are important in driving prostate cancer. It therefore shows considerable promise as a novel precision therapy for prostate cancer.

Conclusions

In conclusion, we have shown that OCT1002 can control the growth of CRPC xenografted PC3 tumours, in a similar way to that observed in androgen-sensitive LNCaP tumours. At a single dose of OCT1002 the effects compared favourably to the multiple dosing schedules of abiraterone, cabazitaxel and docetaxel in these prostate cancer models. Our DSF studies showed the promise of using OCT1002 in combination with other drugs to inhibit tumour growth and significantly diminish

tumour vasculature density. Together, our results provide the first evidence for the potential for OCT1002 to improve treatment responses in CRPC, especially where it is shown that patient tumours are hypoxic, a feature which is widely reported for this tumour type.

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DISCLOSURE STATEMENT

Conflicts of interest: SR McKeown and PJ Smith are directors of OncoTherics Ltd; RJ Errington, LH Patterson and PJ Smith are directors of Biostatus Ltd. J Worthington and SR McKeown are directors of AxisBioservices. H Nesbitt and DJ McKenna declare no conflict of interest.

TITLES AND LEGENDS TO FIGURES

Figure 1. OCT1002 provides equivalent tumour growth control to clinically approved treatments in hypoxic PC3 xenograft model

(A) Oxygenation, prior to treatment, of murine prostate tumour xenografts (LNCaP, 22Rv1, PC3) in nude mice (each n ≥ 5) measured with an Oxylite oxygen electrode (B) Effect of OCT1002 on weight of nude mice (vehicle, 10 or 30 mg/kg OCT1002). (C) Nude mice were implanted with $2x10^6$ PC3 cells. When tumours reached $150mm^3$ mice were treated for 28 days with: vehicle (0.1% DMSO in corn oil), cabazitaxel (5mg/kg; 1x week), abiraterone (98 mg/kg; daily), docetaxel (10mg/kg; 2x week), OCT1002 (30mg/kg; day 1) or OCT1002 (30mg/kg; day 1 and day 7). Tumour volumes were measured every 2 days. Inset table shows statistical results from 2way ANOVA analysis used to compare tumour growth with each treatment to Vehicle treatment at each time point (p-values:*p<0.05, ***p*<0.01, ****p*<0.001, ns; non-significant). (D) Experiment as for 2C except that all treatments were stopped at day 14 and tumour growth was monitored for a further 28 days**.** OCT1002 was given only as a single dose on day 1. (E) Representative images of mice and excised tumours on day 42 from treatment groups described in 2D.

Figure 2. OCT1002 inhibits tumour growth and vascularisation in a dorsal skin fold PC3 model.

The dorsal skin fold (DSF) window chamber was sutured onto the back of nude mice. A PC3 tumour fragment from a donor xenograft was placed in the chamber and left for one week to allow vascularisation. Dosing then commenced (day 0); vehicle (0.1% DMSO in corn oil), cabazitaxel (5mg/kg; 1x week), abiraterone (98 mg/kg; daily), docetaxel (10mg/kg; 2x week), OCT1002 (30mg/kg; day 1). Images were taken once per week. (A) and (B) PC3 tumour growth in DSF chamber. (C) Quantification of vessel coverage during treatment analysed from images, as detailed in the methods. For A-C, data is expressed as the mean ± SEM (n ≥ 3) relative to control at each time

point. (D) Representative images of tumour size and vasculature. (Student t-test p-values: *p<0.05, **p<0.01).

Figure 3. PC3 xenograft tumours treated *in vivo* with OCT1002 in combination with standard prostate cancer chemotherapeutics.

Nude mice were implanted with $2x10^6$ PC3 cells. When tumours reached $150mm^3$ they were treated during the first 14 days with doses as described in Figure 1, in the presence or absence of OCT1002 (30mg/kg; day 1). After 14 days, treatment was stopped. Tumour volumes were measured every 2 days for 42 days. No significant differences were noted between combination and single therapies.

Figure 4. Effect of OCT1002 in combination with standard prostate cancer chemotherapeutics on PC3 tumour growth in dorsal skin fold model

The DSF model was initiated as described in Figure 2. OCT1002 (30mg/kg; day 1) was administered alone or combination with (A) abiraterone (B) cabazitaxel or (C) docetaxel at the respective doses described in Figure 2. Images were taken once per week. Tumour surface area data is expressed as the mean ± SEM (n ≥ 3) relative to control at each time point. In each graph student t-test was used to compare combination to OCT1002 alone (p-values:*p<0.05, **p<0.01, ***p<0.001) and to respective standard drug monotherapy (p-values: ^p<0.05, ^^p<0.01, ^^^p<0.001).

Fig. 5 Effect of OCT1002 in combination with standard prostate cancer chemotherapeutics on PC3 tumour vascularisation in dorsal skin fold model

The DSF model and dosing as described in Figure 4. Tumour vessel coverage data is expressed as the mean ± SEM (n ≥ 3) relative to control at each time point. In each graph student t-test was used to compare combination to OCT1002 alone (p-values:*p<0.05, **p<0.01, ***p<0.001) and to respective standard drug monotherapy (p-values: ^p<0.05, ^^p<0.01, ^^^p<0.001).

Fig 1.

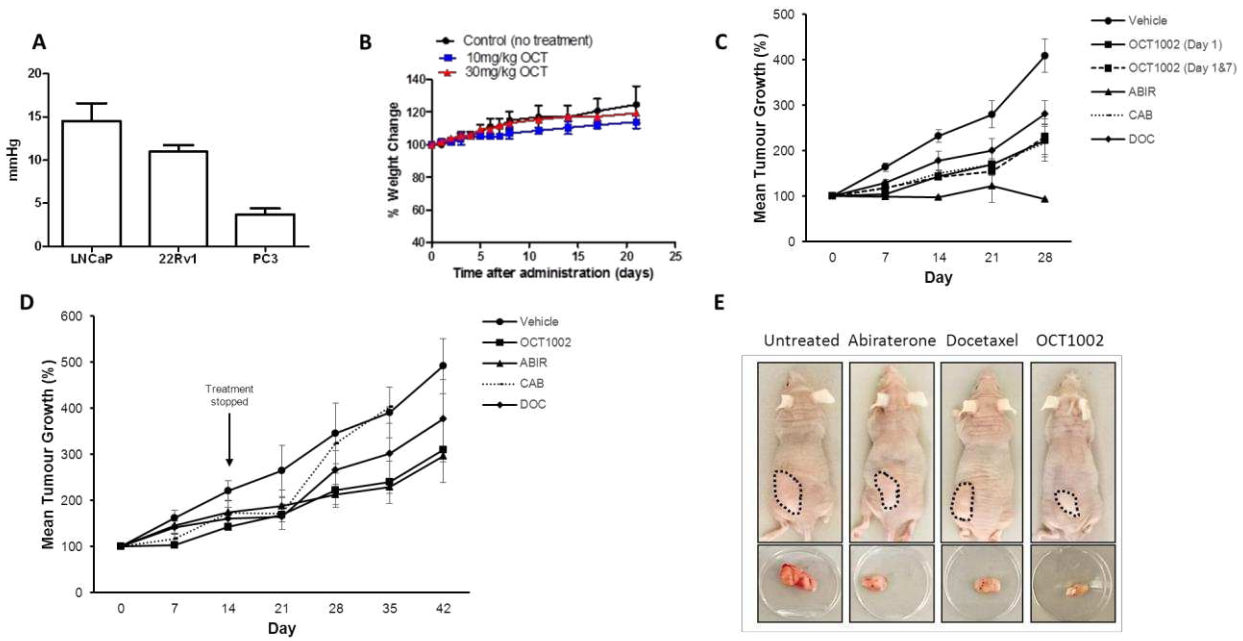
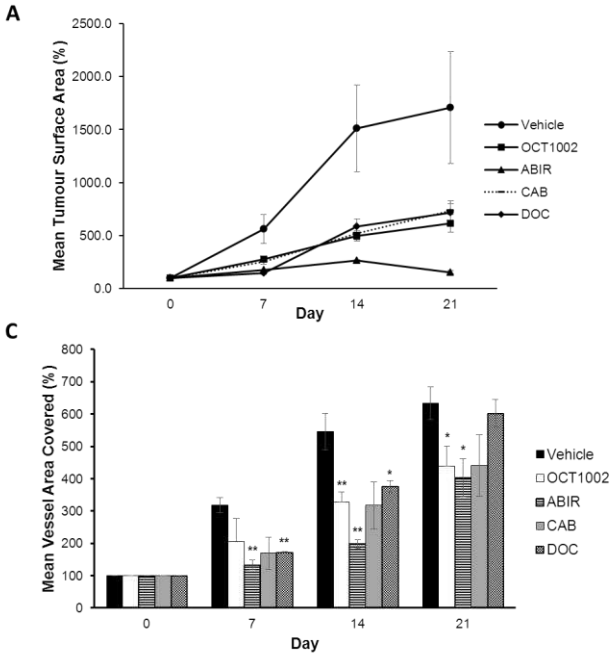


Fig 2.



B

	Day 7	Day 14	Day 21
Vehicle	560 ± 138.13	1513.6 ± 409.6	1707.9 ± 526.9
OCT1002	274.1 ± 25.2	496.8 ± 46.8 *	613.3 ± 83.8 *
Abiraterone	172.7 ± 2.23 *	266.2 ± 4.7 *	152.1 ± 16 *
Cabazitaxel	254.1 ± 23	520.8 ± 62.9	732.1 ± 95.4
Docetaxel	142.9 ± 11 *	587.2 ± 66.3	716.3 ± 81.8

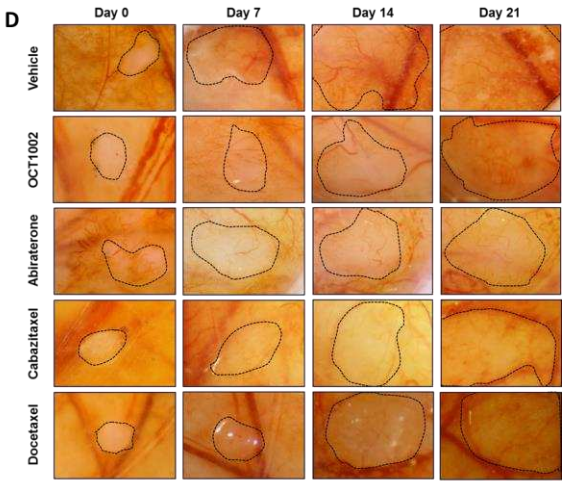


Fig 3.

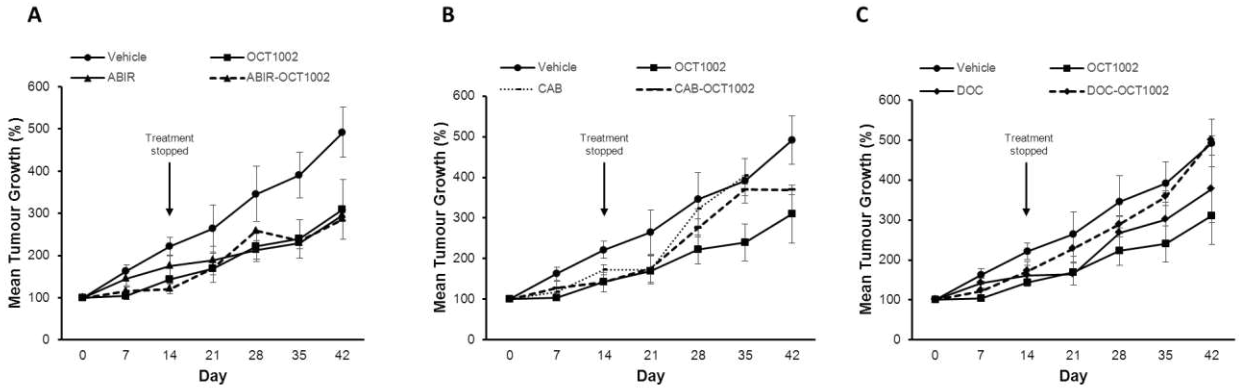
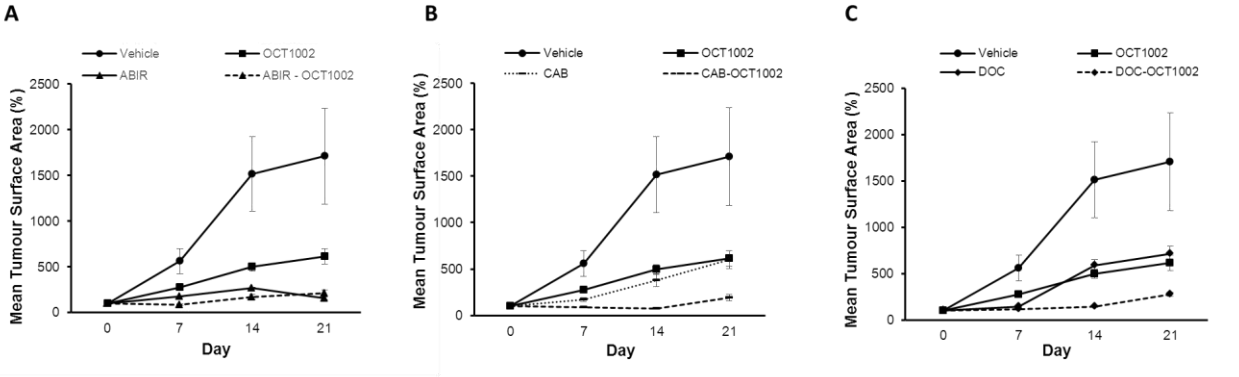


Fig 4.

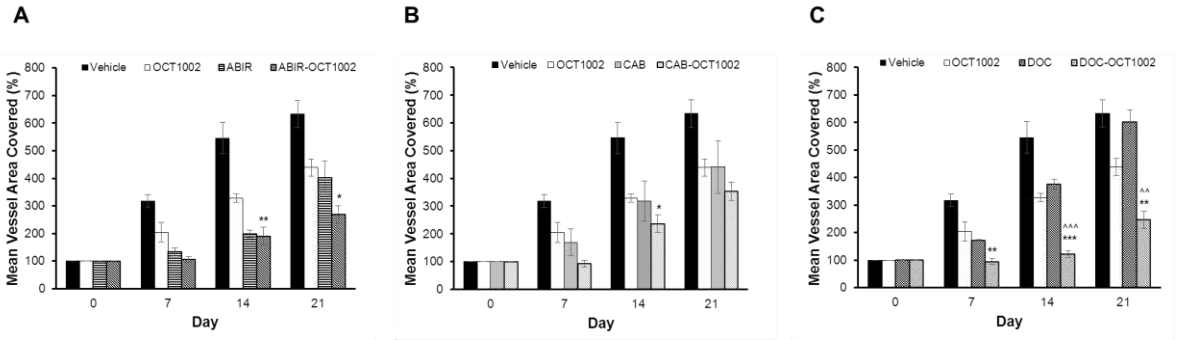


	Day 7	Day 14	Day 21
Vehicle	560 ± 138.13	1513.6 ± 409.6	1707.9 ± 526.9
OCT1002	274.1 ± 25.2	496.8 ± 46.8	613.3 ± 83.8
Abiraterone	172.7 ± 2.23	266.2 ± 4.7	771 ± 128.5
Abiraterone + OCT1002	83.7 ± 5.3 ** ^^^	167.3 ± 24.8 ** ^	208 ± 37.1 *

	Day 7	Day 14	Day 21
Vehicle	560 ± 138.13	1513.6 ± 409.6	1707.9 ± 526.9
OCT1002	274.1 ± 25.2	496.8 ± 46.8	613.3 ± 83.8
Cabazitaxel	254.1 ± 23	520.8 ± 62.9	732.1 ± 95.4
Cabazitaxel + OCT1002	85.1 ± 6.6 ** ^^	74 ± 7.9 *** ^^	193 ± 36 ** ^^

	Day 7	Day 14	Day 21
Vehicle	560 ± 138.13	1513.6 ± 409.6	1707.9 ± 526.9
OCT1002	274.1 ± 25.2	496.8 ± 46.8	613.3 ± 83.8
Docetaxel	142.9 ± 11	587.2 ± 66.3	716.3 ± 81.8
Docetaxel + OCT1002	117.1 ± 6.9 **	142.8 ± 7.7 ** ^^	274.4 ± 22.3 * ^^

Fig 5.



Supplementary Fig. 1